

Presence of nanobacteria in psammoma bodies of ovarian cancer: evidence for pathogenetic role in intratumoral biomineralization

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Aims: The presence of laminated, calcified extracellular debris known as psammoma bodies is a well-known histomorphological feature of ovarian adenocarcinomas and other human malignancies. Biomineralization has recently been found to be associated with a group of extremely small Gram-negative bacteria capable of precipitating calcium salts. The aim of the present study was to evaluate a possible pathogenic link between the development of psammoma bodies and nanobacteria infection.

Material and results: Immunohistochemical staining and reverse transcriptase-polymerase chain reaction (RT-PCR) were used to analyse nanobacterial protein and gene expression in eight psammoma body-containing adenocarcinomas and in 10 malignant ovarian tumours without signs of biomineralization. Nanobacterial proteins were detected in eight out of eight

(100%) psammoma-positive tumour samples. Conversely, none of the 10 psammoma-negative tissues (0%) was positive for nanobacterial antigens. Furthermore, nanobacterial mRNA was detectable in all of the four tissues (100%) that contained psammoma bodies, but was absent in all 10 ovarian cystadenocarcinomas (0%) that were psammoma negative.

Conclusions: We found a 100% concordance between the expression of nanobacteria and the presence of psammoma bodies in malignant ovarian tumours. Several lines of evidence suggest the involvement of these organisms in the process of biomineralization. We therefore conclude that nanobacterial infection of malignant ovarian tissue contributes to mechanisms leading to the formation of calcified deposits known as psammoma bodies.

Keywords: biomineralization, nanobacteria, ovarian cancer, psammoma bodies

Introduction

The appearance of onion peel-like calcified structures known as psammoma bodies is a rare but well-known histomorphological feature which can be observed in a variety of human malignancies such as breast cancer,^{1,2} thyroid cancer,^{2,3} meningioma⁴ and endometrial cancer.⁵ In serous adenocarcinomas

of the ovary, the frequent expression of mineralized formations has even led to the term of psammocarcinoma.⁴ However, it should be noted that psammoma bodies do not specifically resemble a histopathological characteristic of neoplastic tissue since these structures have also been identified in benign ovarian cysts, endosalpingiosis and cervical smears of non-malignant origin.^{6,7} Over the last years, several studies have addressed the question of the pathogenic origin of these deposits, which still remains obscure. In a study conducted by Maki and co-workers⁸ utilizing immunohistochemistry and *in-situ* hybridization, the glycoprotein osteopontin

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was identified as a possible factor causing the development of psammoma bodies via the accumulation of calcium phosphate in serous adenocarcinomas of the ovary. Within this, biomineralization and the process of stone formation has been recently associated with the detection of 80–500 nm-sized calcium salt-precipitating organisms named nanobacteria.^{9–11} Although there is much controversy concerning their archetypical origin,¹² these needle-shaped, Gram-negative and filterable particles have been demonstrated to form a calcium phosphate-containing shell, thereby presenting a novel model for tissue calcification.^{9,13–16}

Nanobacteria are able to infect phagocytosing cells via receptor-mediated internalization.^{11,17} They have been shown to exert cytotoxic effects on fibroblasts¹¹ and appear to be involved in the development of kidney stones^{9,10,14} independent of elevated urinary pH values and urease/alkaline phosphatase activity. It has thus been suggested that the biogenic apatite layer present on the cellular surface might act as a nidus promoting the process of crystallization and formation of calcified deposits.^{9,11,14} The hallmark of psammoma bodies includes the presence of small, concentrically laminated calcified debris as a result of biomineralization. In order to gain more information on the pathogenic origin of these structures, the aim of the present study was to examine the possible involvement of nanobacteria in the development of psammoma bodies in ovarian adenocarcinomas.

Materials and methods

PATIENTS AND TISSUE SPECIMENS

Malignant tumour tissue was obtained from 18 patients with primary serous papillary adenocarcinoma during surgical removal of the malignant tumour. The University of Vienna IRB approved the study and all patients had given written informed consent prior to inclusion in the study. In each case, the tumours were bisected and one aliquot of tumour tissue was snap-frozen within 3 min of surgical resection for gene expression analysis. Another aliquot was formalin-fixed and paraffin-embedded for histological diagnosis and immunohistochemical analysis.

Patients were aged between 40 and 64 years with a median age of 56 years. Patients with a history of prior chemotherapy, pelvic inflammatory disease, second malignant disease and prior surgery of the ovaries were excluded from the study. Eight out of 18 patients exhibited microscopically detectable intratumoral calcifications, thereby fulfilling the

criteria of psammoma bodies. Ten tumours in which psammoma bodies were undetectable in haematoxylin and eosin (H&E) sections were used as controls. Of the 18 tissue specimens, four were classified as well differentiated, eight were classified as moderately differentiated and six as poorly differentiated serous adenocarcinomas.

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Paraffin sections (5 µm) were de-paraffinized and endogenous peroxidase was inactivated by a 15-min treatment with methanol containing 0.6% hydrogen peroxide. Additional antigen retrieval was achieved by immersion of the sections in 10 mM of citrate buffer (pH 6.0) and boiling for 30 min in a microwave pressure cooker. After a short rinsing step in tap water, non-specific binding was blocked with goat serum for 30 min. A monoclonal mouse antibody against nanobacteria (monoclonal mouse antibody, A4002; Nanobac Oy, Kuopio, Finland) was added at a concentration of 1 : 50. The primary antibody was omitted in negative controls and substituted by isotype immunoglobulin. All slides were then incubated at room temperature for 3 h, washed in PBS for 5 min, and incubated with peroxidase-labelled dextran polymers conjugated to antimouse immunoglobulins (EnVisionTM; DakoCytomation, Glostrup, Denmark). The sections were subsequently washed with PBS and subjected to 3-amino-9-ethylcarbazole (AEC) substrate for 10 min. Rinsing in distilled water for 5 min stopped the enzymatic reaction. All slides were counterstained with Mayer's haematoxylin, dehydrated and mounted with p-Xylene-bis(N-pyridinium bromide) (DPX) mounting medium (FLUKA, Buchs, Switzerland). Formalin-fixed glass slides with immobilized nanobacteria were used as positive controls (nanobacteria culture I2001; Nanobac Oy).

EXTRACTION OF NANOBACTERIA AND REVERSE TRANSCRIPTION

Nanobacteria cultures (Strain Seralab 901045; Nanobac Oy) were cultivated in Dulbecco's modified Eagle's medium for 8 weeks before being carefully scraped off the surface of the biofilm under sterile conditions. Also, 14 solid tissues from adenocarcinomas of the ovary (four fresh frozen adenocarcinomas of the ovary containing psammoma bodies; 10 ovarian adenocarcinomas lacking psammoma bodies—negative controls) were frozen under liquid nitrogen and powdered with a pestle. Approximately 5–10 µg of powdered tumour tissue (>80% of epithelial cells) as well as the

nanobacterial isolates were then lysed in 500 µl of proteinase K buffer (10 mM Tris pH 7.4; 10 mM EDTA, 150 mM NaCl, 0.4% SDS) and proteinase K (10 mg/ml) for 6 h at 56 °C and then centrifuged for 10 min at 13 000 *g* (Hettich Universal (HU) RF, Tuttlingen, Germany). The supernatant was extracted with phenol/chloroform/isoamylalcohol (25 : 24 : 1), vortexed, and centrifuged again at 13 000 *g* for 5 min followed by a transfer of the aqueous phase to a new tube. Nucleic acids were precipitated with 0.1 vol% of 3 M sodium acetate and 2.5 vol% of 100% ethanol for 10 min on ice followed by another centrifugation at 13 000 *g* for 15 min at 4°C. The supernatant was decanted and the pellet was washed once with 80% ethanol, isolated, dried at room temperature and dissolved in 200 µl of TE-buffer.

Ten microlitres of each bacterial RNA/DNA mix (approximately 0.2–1 µg) were denatured at 65 °C for 5 min and chilled on ice. A master mix consisting of 4 µl of 5 × RT-buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂), 2 µl of dNTPs (10 mM each; Pharmacia, Uppsala, Sweden), 1 µl of RNase inhibitor (20 U/µl; Applied Biosystems, Foster City, CA, USA), 1 µl of reverse primer (20 µM; 'Nano3': 5'-AGT CGC TGA CCC TAC CGT GGT TGC-3'; MWG-Biotech AG, Vienna, Austria), 1 µl dithiothreitol (DTT) and 1 µl of moloney murine leukaemia virus reverse transcriptase (MMLV-RT; 200 U/µl; Amersham Bioscience UK Ltd, Little Chalfont, UK) were added and incubated for 1 h at 42 °C. After inactivation of the reverse transcriptase by incubation at 80°C for 5 min, cDNAs were either processed immediately for amplification or stored at -20°C.

POLYMERASE CHAIN REACTION AMPLIFICATION OF NANO BACTERIAL NUCLEIC ACID

The reverse transcription products (10 µl) were mixed with a polymerase chain reaction (PCR) master mix, consisting of 10 × PCR buffer (15 mM MgCl₂, 500 mM KCl, 100 mM Tris-HCl, pH 8.3), 100 mM of each dNTP, and 20 µM of 'Nano5' primer (5'-ATG CAA GTC GAG CGC CCC GCA AGG-3') and 20 µM of the reverse primer ('Nano 3' both MWG-Biotech AG) and Taq-DNA polymerase (2.5 U). Thirty cycles were carried out at 94 °C, 68 °C and 72 °C, each for 2 min, with an extension of 5 s in each following cycle. The RT mix was replaced by ddH₂O in negative controls. The PCR products were stored at -20 °C until ready for electrophoresis. Then the PCR mix (10 µl) was subjected to 0.2% agarose gel electrophoresis at 80 V for 30 min and nucleic acid bands were visualized by ethidium bromide staining.

Results

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In eight out of eight (100%) cases, psammoma bodies exhibited a clearly visible positive staining reaction with the anti-nanobacteria antibody (Figure 1A,B). As a rare event, a weak positive staining was detected in the cytoplasm of some malignant epithelial cells, but was absent in all other tissue components including tumour stroma. Negative controls and all 10 samples without any evidence of psammoma bodies lacked positive staining for nanobacteria (Figure 1C). As expected, a strong positive reaction was observed in the cell cultures of nanobacteria that were used as positive controls.

RT-PCR

In order to detect nanobacterial DNA in psammoma body-containing ovarian cancer tissues, RT-PCR was performed with primers specifically designed for the detection of nanobacterial genomic elements. All four tissue samples, in which psammoma bodies were present and in which nanobacteria were detected by immunohistochemistry, also exhibited nanobacterial mRNA (band at 1373 bp in Figure 2A). By contrast, none of the 10 ovarian adenocarcinomas that did not exhibit psammoma bodies in H&E sections examined by light microscopy and immunohistochemistry exhibited bands corresponding to the presence of bacterial mRNA (Figure 2B), thereby demonstrating the specificity of nanobacteria being exclusively present in tissue containing psammoma bodies.

Discussion

The formation of crystalline calcium phosphate serving as a nidus for the development of extracellular debris is preceded by the liberation of phosphate originating from cell membranes through phosphatase activity and electrostatic binding of calcium.¹⁸ Although the microbial origin of nanobacteria is still controversial,^{12,19} these organisms have been demonstrated to accumulate these two compounds directly on their surfaces, thereby linking the process of biomineralization and tissue calcification to the presence of these organisms.^{11,13} We speculated whether nanobacteria might also serve as a possible cause for the formation of psammoma bodies that are commonly observed in adenocarcinomas of the ovary. Indeed, we were able to identify nanobacterial antigens using immunohistochemistry on paraffin-embedded tissue sections of

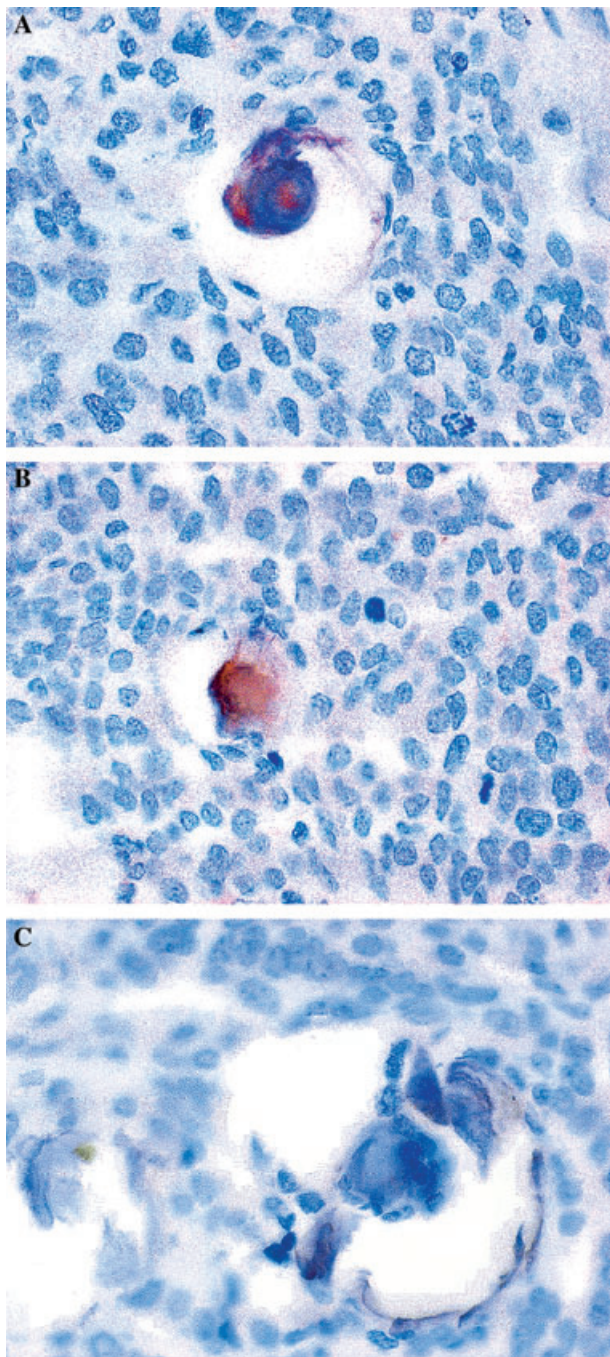


Figure 1. A,B, Positive immunohistochemical staining for nanobacterial antigens in spherical, laminated calcifications fulfilling the criteria for psammoma bodies present in adenocarcinoma of the ovary. A tissue section that also exhibited positive staining for nanobacteria was used as a negative control where the primary antibody was substituted by isotype immunoglobulin (C).

tumours that exhibited psammoma bodies. In contrast, tumours negative for psammoma bodies did not exhibit a positive staining reaction. To confirm these findings, RT-PCR was applied to RNA extracted from frozen

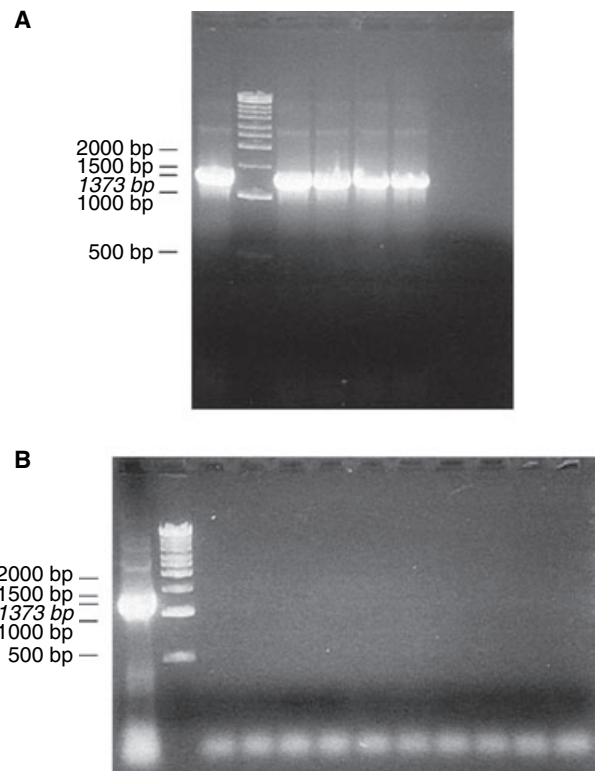


Figure 2. A, Reverse transcriptase-polymerase chain reaction for nanobacterial 16S ribosomal RNA. Positive control representing nanobacterial RNA isolated from nanobacteria stock culture (lane 1); size marker (lane 2); nanobacterial RNA products (1373 bp) deriving from four fresh frozen adenocarcinomas of the ovary positive for psammoma bodies (lanes 3–6). Ovarian adenocarcinomas lacking psammoma bodies were used as negative controls (lanes 7 and 8). B, Additional negative controls representing ovarian adenocarcinomas without expression of psammoma bodies (lane 1, positive control; lane 2, size marker; lanes 3–12, the expression of a specific ribosomal RNA fragment of nanobacterial RNA is absent).

tumour specimens. Indeed, nanobacterial gene expression was detected in the psammoma body-containing tumours.

Nanobacteria have been detected in bovine and human blood¹¹ and have been shown to pass the renal glomerulum as living, slowly multiplying organisms.^{9,11} Recently, Breitschwerdt and colleagues²⁰ were able to detect nanobacterial antigens in beef, suggesting that bacteraemia and cellular infection might occur via the oral intake of these organisms. Although there is no direct evidence for nanobacterial-related disease in humans, it should be kept in mind that nanobacteria do exert cytotoxic effects on mammalian cells *in vitro*^{9,13} and possess the ability to cross the placenta.¹³ Unfortunately, nanobacteria are barely detectable with standard microbiological methods because they are difficult to stain and are able to pass

through 0.1- μ m filters. Several lines of evidence indicate that nanobacterial infections provide an alternative mechanism for induction of biomineralization and are responsible for the formation of extracellular calcification.^{9–11,14} Psammoma bodies contain mucopolysaccharides, iron and, as a typical feature of these structures, calcium salts.⁸ The present study provides evidence for a causative link between the presence of nanobacterial infections and the development of psammoma bodies in ovarian adenocarcinomas. To our knowledge, this is the first report demonstrating the presence of nanobacterial genomic elements in malignant ovarian tissues containing psammoma bodies. We hypothesize that nanobacteria might not only promote the biomineralization process in the genitourinary tract, but also lead to the development of calcified deposits known as psammoma bodies in human malignancies such as ovarian cancer.

References

- Hirota S, Ito A, Nagoshi J *et al*. Expression of bone matrix protein messenger ribonucleic acids in human breast cancers. Possible involvement of osteopontin in development of calcifying foci. *Lab. Invest.* 1995; **72**: 64–69.
- Tunio GM, Hirota S, Nomura S, Kitamura Y. Possible relation of osteopontin to development of psammoma bodies in human papillary thyroid cancer. *Arch. Pathol. Lab. Med.* 1998; **122**: 1087–1090.
- Ellison E, Lapuerta P, Martin SE. Psammoma bodies in fine-needle aspirates of the thyroid: predictive value for papillary carcinoma. *Cancer* 1998; **84**: 169–175.
- Hirota S, Nakajima Y, Yoshimine T *et al*. Expression of bone-related protein messenger RNA in human meningiomas: possible involvement of osteopontin in development of psammoma bodies. *J. Neuropathol. Exp. Neurol.* 1995; **54**: 698–703.
- Zreik TG, Rutherford TJ. Psammoma bodies in cervicovaginal smears. *Obstet. Gynecol.* 2001; **97** (5 Part 1): 693–695.
- Hallman KB, Nahhas WA, Connelly PJ. Endosalpingiosis as a source of psammoma bodies in a Papanicolaou smear. A case report. *J. Reprod. Med.* 1991; **36**: 675–678.
- Qazi FM, Geisinger KR, Barrett RJ, Hopkins MB 3rd, Holleman IL Jr. Cervicovaginal psammoma bodies. The initial presentation of the ovarian borderline tumor. *Arch. Pathol. Lab. Med.* 1988; **112**: 564–566.
- Maki M, Hirota S, Kaneko Y, Morohoshi T. Expression of osteopontin messenger RNA by macrophages in ovarian serous papillary cystadenocarcinoma: a possible association with calcification of psammoma bodies. *Pathol. Int.* 2000; **50**: 531–535.
- Ciftcioglu N, Bjorklund M, Kuorikoski K, Bergstrom K, Kajander EO. Nanobacteria: an infectious cause for kidney stone formation. *Kidney Int.* 1999; **56**: 1893–1898.
- Dorrell S. Nanobacteria linked to kidney disease. *Mol. Med. Today* 1999; **5**: 373.
- Kajander EO, Ciftcioglu N. Nanobacteria: an alternative mechanism for pathogenic intra- and extracellular calcification and stone formation. *Proc. Natl Acad. Sci. USA* 1998; **95**: 8274–8279.
- Cisar JO, Xu DQ, Thompson J, Swaim W, Hu L, Kopecko DJ. An alternative interpretation of nanobacteria-induced biomineralization. *Proc. Natl Acad. Sci. USA* 2000; **97**: 11511–11515.
- Kajander EO, Ciftcioglu N, Aho K, Garcia-Cuerpo E. Characteristics of nanobacteria and their possible role in stone formation. *Urol. Res.* 2003; **31**: 47–54.
- Kajander EO, Ciftcioglu N, Miller-Hjelle MA, Hjelle JT. Nanobacteria: controversial pathogens in nephrolithiasis and polycystic kidney disease. *Curr. Opin. Nephrol. Hypertens.* 2001; **10**: 445–452.
- Benzerara K, Menguy N, Guyot F, Dominici C, Gillet P. Nanobacteria-like calcite single crystals at the surface of the Tataouine meteorite. *Proc. Natl Acad. Sci. USA* 2003; **100**: 7438–7442.
- Ciftcioglu N, McKay DS, Kajander EO. Association between nanobacteria and periodontal disease. *Circulation* 2003; **108**: e58–59; author reply e58–59.
- Kim BH, Park HS, Kim HJ *et al*. Enrichment of microbial community generating electricity using a fuel-cell-type electrochemical cell. *Appl. Microbiol. Biotechnol.* 2004; **63**: 672–681.
- Morgan MB. Nanobacteria and calcinosis cutis. *J. Cutan. Pathol.* 2002; **29**: 173–175.
- Drancourt M, Jacomo V, Lepidi H *et al*. Attempted isolation of *Nanobacterium* sp. microorganisms from upper urinary tract stones. *J. Clin. Microbiol.* 2003; **41**: 368–372.
- Breitschwerdt EB, Sontakke S, Cannedy A, Hancock SI, Bradley JM. Infection with *Bartonella weissii* and detection of *Nanobacterium* antigens in a North Carolina beef herd. *J. Clin. Microbiol.* 2001; **39**: 879–882.